

A LOOK AT

# MICROBIAL METABOLISM

**M**icroorganisms are the most abundant life form on earth — both in the number of species and quantity and weight of living organisms. They have a history spanning over 3.5 billion years and have evolved to adapt to a wide range of environmental conditions and to survive with diverse sources of carbon and energy. Microorganisms are so named because they are usually too small to see with the naked eye (which is about a tenth of a millimeter).

In a typical gram of sediment, there are thousands of species of microorganisms and billions of individual organisms. In soils and sediments, microbes play a key role in the degradation of stems, leaves, and roots of plants — leading to the endless cycling of carbon and nitrogen between the atmosphere and terrestrial biosphere. Microorganisms are also present at great depths below the land surface. Recent studies have shown that aquifers and oil reservoirs are inhabited by a diverse assortment of microorganisms that have learned to live in harsh conditions where it is hot, salty, and food is in short supply.

Microorganisms span the three domains of life: Bacteria, Eukarya, and the recently recognized Archaea. These three domains are divided according to the structure of their cells. The cells of higher animals and plants are eukaryotic and have a true nucleus. The ancestors of multicellular organisms are eukaryotic microorganisms. Eukaryotic microorganisms include algae, fungi, and protozoa. Bacteria and archaea, however, do not have a discrete nucleus and are called, collectively, prokaryotes. Most prokaryotes are one-celled organisms, whereas eukaryotes may be one-celled or more complex, multicellular organisms.<sup>1</sup>

Microorganisms also can be categorized according to their respiratory metabolic processes and sources of nutrition. This classification can be used to characterize their bioremediation potential. Some microorganisms, aerobes, require oxygen to grow, while others, anaerobes, are able to grow in environments devoid of available oxygen. Some organisms will grow on the simplest sources of carbon such as methane, while others will only grow on more complex carbon substrates. In sediment and groundwater systems, there is a large diversity of organic molecules that can provide a source of carbon for microbial growth. In addition to carbon, microorganisms also need electron donors and acceptors. Some metals and radionuclides can act as these donors and acceptors. Enzymatically catalyzed transfer of electrons (by oxidation and reduction reactions) between donors and acceptors releases energy for carrying out biochemical reactions. Microbial metabolism can play an important part in transformations of metals and radionuclides, changing the form, or speciation, of these contaminants.

Bioremediation is a technology that uses metabolic processes to degrade or transform contaminants so that they are no longer in a harmful form. In some cases the contaminant is a primary part of the metabolic process, acting as the main source of carbon and energy for the cell. In others, it is transformed while a second substance serves as a primary energy or carbon source. This cometabolism process may be purely fortuitous, and the microorganism gains nothing from the process. Contaminant degradation may result in daughter products that can be metabolized or in ones that persist.

Transformation of metals and radionuclides proceeds somewhat differently. Although they cannot be sources of carbon, metals and radionuclides can provide energy, and they can also

1. For more information about how scientists identify microorganisms, see the feature “Who’s Out There? Identifying Microbial Species that Live in the Subsurface” on page 29.

be transformed indirectly in the energy transfer process. Metals and radionuclides can be transformed directly through changes in valence state by acting as electron donors or acceptors, or by acting as co-factors to enzymes. They can also be transformed indirectly by reducing and oxidizing agents produced by the microorganism that cause changes in pH or redox potential.

Transformation may also occur when microorganisms produce chelating agents that bind the metal or radionuclide or degrade the chelating agent, or when the microorganism produces surfactants that desorb metals from sediments. The goal of this section is to introduce the reader to some of the basic metabolic processes involved in biotransformation of metals and radionuclides.<sup>2</sup>

## BASIC MICROBIAL METABOLIC PROCESSES

Metabolism consists of the sequences of biochemical reactions, or pathways, in an organism that result in activity, growth, and reproduction. These include degradative (catabolic) and constructive (anabolic) processes. Catabolic processes break down larger molecules into simpler components, producing energy for microbial growth and reproduction. Contaminants can be transformed into less harmful forms or degraded completely (mineralized) to inorganic components through these catabolic processes. Some of the most important components of catabolism are nutrient and energy sources; microbial respiration; basic respiratory oxidation–reduction reactions, which generate energy and transfer electrons from electron donors to electron acceptors; and enzymes, which serve as catalysts to these reactions.

### Nutrient Sources

Carbon, nitrogen, and phosphorus are the major nutrients needed by the cell. This is because they are the basic elemental components of the proteins, sugars, and nucleic acids that comprise the cell. Organisms that require an organic or complex source of carbon are called heterotrophs. Those that use inorganic sources of carbon like carbon dioxide ( $\text{CO}_2$ ) are called autotrophs.

Most microorganisms need nitrogen because it is a major constituent of proteins and nucleic acids. Nitrogen can be found in nature in both organic and inorganic forms. However, the most abundant forms of nitrogen in nature are inorganic — either ammonia ( $\text{NH}_3$ ), nitrate ( $\text{NO}_3^-$ ), or nitrogen gas

( $\text{N}_2$ ). Most microbes can use either ammonia or nitrate as their sole nitrogen source. Nitrogen-fixing bacteria can use  $\text{N}_2$  gas as a nitrogen source, fixing it directly from the air.

Microorganisms also need other nutrients, although to a lesser extent. Production of ATP (adenosine triphosphate — the principal energy carrier molecule of the cell) and synthesis of nucleic acids and phospholipids require phosphorus, which occurs in nature in the form of organic and inorganic phosphates ( $\text{PO}_4^{3-}$ ). The amino acids cysteine and methionine require sulfur. Most sulfur originates from inorganic sources, usually sulfate ( $\text{SO}_4^{2-}$ ) or hydrogen sulfide ( $\text{H}_2\text{S}$ ). Several enzymes need potassium, including some that are involved in protein synthesis. Potassium occurs in nature inorganically in the form of salts. Magnesium stabilizes ribosomes, cell membranes, and nucleic acids. Cells need iron in large amounts as it plays a major role in cellular respiration — it is a key component of the cytochromes and iron-sulfur proteins involved in electron transport. Most inorganic iron is highly insoluble, so many organisms produce specific iron-binding agents called siderophores, which solubilize iron salts and transport iron into the cell. Iron is found inorganically as Fe(III), Fe(II), and Fe(0) (elemental iron).

### Energy Sources

Microorganisms can use two sources of energy other than organic compounds — light and inorganic chemicals. Those that use light are phototrophs, converting that light energy to

2. To learn more about microbial metabolism, see *Brock Microbiology of Microorganisms* (Madigan et al., 1997).

chemical energy through photosynthesis; those that use chemicals are chemotrophs. Although many organisms obtain their energy from light, most microbes are chemotrophs. Microorganisms that use metals and radionuclides as primary sources of energy are chemolithotrophs, that is, they use inorganic chemical compounds as an energy source.

### Microbial Enzymes Acting as Catalysts

Enzymes are proteins that catalyze chemical reactions in the cell. One of the most important of these reactions is oxidation–reduction (redox) in catabolic metabolism. These redox reactions transfer electrons and release energy from a substance. The substance that an enzyme acts upon is called the reactant, or substrate. This is often the contaminant in bioremediation. A specific enzyme-catalyzed reaction is usually only one of many of the reactions in a catabolic or anabolic pathway.

For a reaction to even occur, molecules must first reach a reactive state in order for chemical bonds to be broken. The amount of energy required to bring all molecules in a chemical reaction to the reactive state is called the activation energy. Once activation has occurred, the reaction can then proceed.

Catalysts are the substances that activate reactants. They do this by lowering the amount of activation energy needed to initiate a reaction. They also increase the rate at which a reaction will occur. However, they are not themselves changed by the reaction. Enzyme-catalyzed reactions occur very quickly. Enzymes can increase the rate of chemical reactions from  $10^8$  to  $10^{20}$  times what would occur spontaneously.

Some enzymes are highly specific in the reactions or groups of reactions they catalyze. In an enzyme-catalyzed reaction, the enzyme (E) temporarily combines with the reactant, or substrate (S), in an enzyme–substrate complex. The reaction occurs and the product (P) is released. This product is the transformed — oxidized or reduced — substrate. Then the enzyme returns to its original state:



The combination of enzyme and substrate usually depends on weak bonds to join the enzyme to the substrate. To catalyze a reaction, an enzyme must bind the correct substrate and position it correctly on the enzyme's active site. This places a strain on specific bonds in the substrate, which causes the substrate to break into component products. The result of this enzyme–substrate complex formation is a reduction in the activation energy required to make the reaction occur and transform the substrate. Enzymes are named for the substrate they bind or the chemical reaction they catalyze, denoted by “ase” at the end of the name. For example, ribonuclease is an enzyme that decomposes ribonucleic acid.

### Oxidation–Reduction

Microorganisms obtain nutrients and energy for cellular processes and growth through oxidation–reduction reactions, which are catalyzed by specific enzymes. Oxidation–reduction, or redox, reactions involve the transfer of electrons from one reactant to another.<sup>3</sup> This transfer occurs through the donation of one or more electrons from an energy source (substrate), called the electron donor, and accepted by the electron acceptor, leading to changes in the chemical state of both donor and acceptor. In a redox reaction, the electron donor is oxidized and the electron acceptor is reduced. Because electrons cannot exist alone in solution, but only as parts of atoms or molecules, an oxidation cannot occur without a subsequent reduction.

In biochemistry, redox reactions often involve the transfer of not just electrons but hydrogen atoms. When the electron is removed, the hydrogen atom becomes a proton (or positive hydrogen ion,  $H^+$ ).

In the oxidizing half-reaction  $H_2 \rightarrow 2e^- + 2H^+$ , the electron donor, hydrogen gas ( $H_2$ ), is oxidized as it releases two electrons and two protons.

In a second reducing half-reaction, the oxidation of  $H_2$  can be coupled to the reduction of

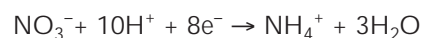
3. See the feature “Opposites Attract: Valences, Bonds, and Redox Reactions” in Section III.

**Table 4.1.**  
**Microbially Significant Half-Reaction**  
**Reduction Potentials**

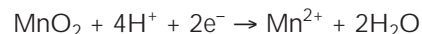
Redox Pairs	E <sub>0</sub> (V)
$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$	+1.229 <sup>*</sup>
$MnO_2(s) + 4H^+ + 2e^- \rightarrow Mn^{2+} + 2H_2O$	+1.208 <sup>*</sup>
$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$	+0.94 <sup>†</sup>
$Fe^{3+} + e^- \rightarrow Fe^{2+}$	+0.77 <sup>*</sup>
$SO_4^{2-} + 4H^+ + 2e^- \rightarrow H_2SO_3 + H_2O$	+0.20 <sup>*</sup>
$2H^+ + 2e^- \rightarrow H_2$	0.0 <sup>†</sup>

<sup>\*</sup> Oxtoby et al., 1994; <sup>†</sup> Tinoco et al., 1985.

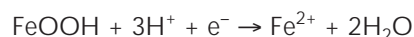
a well-defined sequence of redox reactions that occurs (Sposito, 1989). First, nearly all of the  $O_2$  is consumed by the reaction described above. When the  $O_2$  is nearly depleted, nitrate ( $NO_3^-$ ) is reduced to  $NO_2^-$ ,  $NH_4^+$ ,  $N_2O$ , and  $N_2$  by reactions such as:



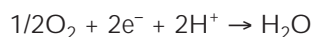
Complete reduction of nitrate to  $N_2$  is commonly referred to as denitrification. Manganese reduction, leading to the dissolution of solid phase magnesium oxide, can begin while nitrate is present, by the reaction:



After nitrate is depleted, dissolution of  $Fe^{3+}$  minerals to aqueous  $Fe^{2+}$  occurs by reactions such as:



the electron acceptor  $O_2$ .



The net oxidation–reduction reaction is balanced:



The tendency for a substance to donate or accept electrons is expressed by its reduction potential ( $E_0$ ). Substances with large positive reduction potentials readily accept electrons. Substances with lower or negative reduction potentials readily give up electrons. Table 4.1 lists the reduction potentials for some of the most important redox half-reactions for bioremediation of metals and radionuclides.

In soil and groundwater systems with abundant carbon and nutrients for microbial activity, there is

Finally, when the potential drops even lower, sulfate reduction becomes the predominant redox process, leading to the formation of reduced forms of sulfur such as  $HS^-$ ,  $H_2S$ , and  $S_2O_3^{2-}$ . Under even more highly reducing conditions, methane is generated by microbial reduction of  $CO_2$  and organic carbon. Because of the ubiquitous occurrence of these common earth elements, redox reactions involving contaminants must be viewed in light of where they lie in this redox sequence and how they compete or combine with these species for electron transfer reactions. Fortunately, as described in Sections V and VI, the reaction products of these major earth elements can also react with some radioactive and metal contaminants to form stable mineral phases.

## MICROBIAL RESPIRATION

Respiration is a fundamental metabolic process whereby microorganisms obtain the energy needed to grow and reproduce. There are two basic divisions of respiration: aerobic and anaerobic.

Aerobic respiration occurs when the terminal electron acceptor is  $O_2$ . Anaerobic respiration is the use of inorganic compounds other than  $O_2$  as terminal electron acceptors.

## WHO'S OUT THERE?

### MICROBIAL SPECIES THAT LIVE IN THE SUBSURFACE

One of the problems that has plagued scientists in bioremediation is how to identify and characterize the microbial communities that live in a contaminated site. Through culturing, microbiologists have been able to grow, at most, one percent of the microbes in a community. Yet even when organisms can be cultured, they cannot always be identified. Over the last few years, however, scientists have developed ways of identifying microbes and assessing the microbial communities in the subsurface.

A community can be assessed directly by isolating DNA and after amplification (see below), determining the sequences of specific genes. After identification, the sequence can be compared to a large database comprising 16S rRNA sequences of previously cultured organisms. The patterns obtained from the fatty acid methyl esters (FAME) of organisms grown under carefully controlled conditions can be used for culture identification. Analogous to the FAME analysis, by carefully identifying specific lipid molecular classes<sup>1</sup> and focusing on the fatty acids of polar phosphate-containing lipids, the community can be further characterized. The total microbial community can be examined, but no one method can furnish a complete analysis. Along with culturing, however, each of these approaches provides a piece of the puzzle.



Figure 4.1. Culture of *Pseudomonas stutzeri* on a plate. Image courtesy of F. Blaine Metting, Pacific Northwest National Laboratory.

**Culturing Microorganisms on Growth Media.** Culturing is a traditional method of identifying a microbial species (Figure 4.1). First a microbial strain representing a single species is isolated from a mixed culture and grown in a sterilized medium in a temperature-controlled incubator. Sugars and amino acids may be added to the medium, as well as some kind of solidifying agent, like agar. Researchers then perform a number of phenotypic tests to identify the cultured organisms by species. With bacteria, the first test will often be a gram stain. This staining is based on a differentiation in cell-wall structure and chemical composition. Gram-negative organisms stain red and gram-positive organisms stain purple. Then the microorganisms are put through further tests, the nature of which depends upon whether they are gram positive or negative, until they are identified by process of elimination. Their FAME patterns and RNA sequences can be used for further confirmation.

**16S rRNA Gene Sequencing.** This identification method can be used with archaea as well as eukarya and bacteria. It is based on determining the phylogenetic position of the unknown microbe among known microorganisms. This determination is based upon a particular DNA strand — its 16S rRNA gene sequence. This sequence is considered the best for these evolutionary measurements because it is highly conserved.

1. Lipids are the organic solvent-extractable, water-insoluble components of cells. These organic molecules are composed of fatty acids and a sugar molecule, usually glycerol.

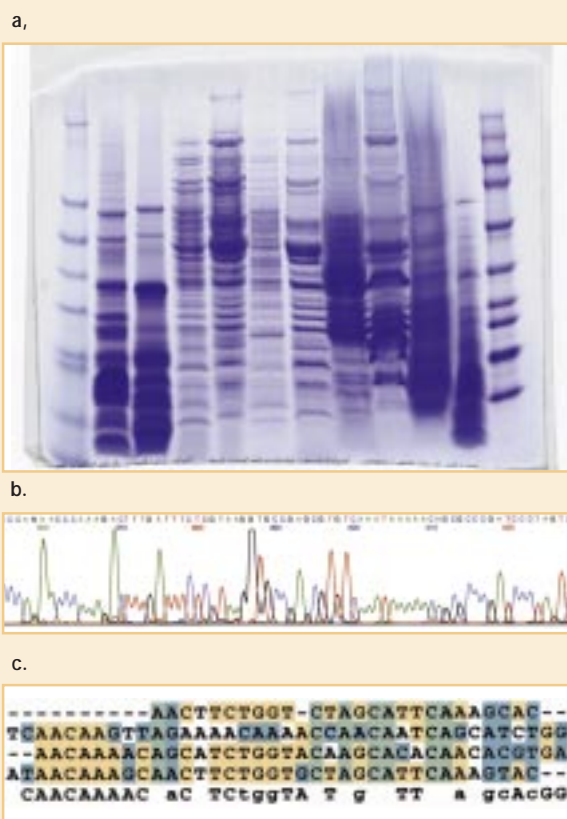


Figure 4.2. (a) RNA sequences are separated by gel electrophoresis. (b) Sequencing results are color coded by base type (adenine — green, guanine — black, cytosine — blue, and thymidine — red). (c) Alignment of four sequences, color coded to denote matching bases. Images courtesy of Tamas Torok, Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory.

Obtaining the 16S rRNA sequence is accomplished in a variety of ways. One of the most common and effective is PCR (polymerase chain reaction),<sup>2</sup> which replicates the 16S rRNA strand. This amplified material is then sequenced. Next, the sequenced 16S rRNA is compared to the sequences of other microorganisms that have been placed in a database created by Carl R. Woese and Gary Olsen at the University of Illinois (the Ribosome Database). Drs. Woese and Olsen have structured all three classes of organisms into relationships with one another based on the differences between the nucleotides in their 16S rRNA strand (Figure 4.2). Pairs of sequences from different organisms are aligned, and the differences in their nucleotide sequences are counted. The number of differences form a basis for measuring the evolutionary distance between organisms. (See the inside back cover for a phylogenetic tree based on the Ribosome Database.) In addition, knowing the phylogenetic position of an unknown, uncultured organism can sometimes allow inference of its physiological properties, which in turn can suggest culture conditions that allow its isolation.

**FAME (Fatty Acid Methylene) Analysis.** This approach is used to identify unknown bacteria through characterization of the fatty acid composition of the lipids in the cell membrane. For the FAME analysis, bacterial cell material is hydrolyzed, and then saponified in sodium hydroxide. The material is then acidified with hydrogen chloride in methanol, causing the fatty acids to be methylated to form methyl esters. The

fatty-acid-methylated esters are then extracted with an organic solvent, and injected into a gas chromatograph. After obtaining the gas chromatogram profile of an isolate, with peak identification by mass spectrometry (Figure 4.3), its FAME profile can be compared to those of known organisms in the FAME database using similarity indexes. The higher the similarity, the more likely the organism matches the database sample. There are only a few thousand species in this database, so identification is limited. However, the database is growing, and as new organisms are cultured their FAME patterns are added.

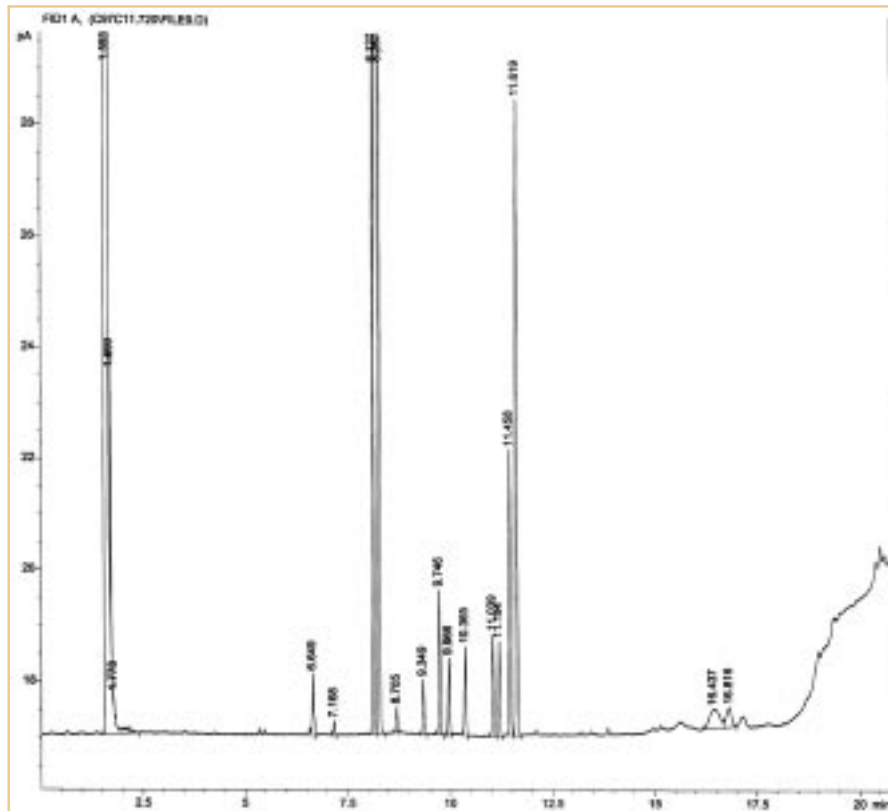
**Signature Lipid Analysis.** This approach is based on extraction of the lipid components of the cells. Extraction results in both a purification and concentration. Of the different lipids extracted, the charged polar phosphate-containing lipids provide insight into the extant community. All living cells are surrounded by a membrane formed of polar lipids. This is the water-resistant barrier between the outside world and the cell. The cells maintain this barrier by constant chemical activity, and when the cells die enzymes in the cells rapidly degrade these lipids so that they lose their charge. Consequently, the total polar lipids are a measure of the living cellular biomass. These polar lipids consist of a three-carbon alcohol glycerol with two fatty acids. The phosphate and other components occupy the third position.

2. A new technology that can now enzymatically amplify minute quantities of specific gene fragments millions of times.

The structures of these polar lipid fatty acids (PLFA) have a great deal of chemical complexity. Therefore, their patterns can be utilized, both in the identification of individual cultured isolates and for characterizing the total microbial community of a given environmental sample. Since most of the organisms in the total sample cannot be cultured, most of the organisms cannot be identified as to species. However, major classes of organisms can be quantitatively identified. The purple staining gram-positive organisms have a PLFA pattern much different than the red staining gram-negative bacteria. Certain groups such as the actinomycetes, the Archea, and the sulfate-reducing bacteria can be identified by their distinct patterns. Higher microbes, such as algae, protozoa, and fungi can also be identified. From shifts in specific lipid patterns induced in cultured organisms by stresses such as starvation, imbalance in nutrients, presence of sublethal toxicants, loss of oxygen, etc., physiological/nutritional status can be determined. Consequently, PLFA analysis provides the viable biomass, composition, and nutritional/physiological status of the community. All of these allow investigators to ask not only who is out there but what the conditions are at the site where bioremediation is to be done. In using PLFA analysis we are “asking the microbes” if the various manipulations are effective. We can then utilize shifts in their ecology as a comprehensive and integrated monitor for toxicity assessment.

Recently, the signature lipid analysis has been expanded in research supported by NABIR by utilization of liquid chromatography/mass spectrometry. This adds much greater specificity and three orders of magnitude in sensitivity. With this technology it is now possible to detect microbes in one well (and at limits of only a few microbes) that were first injected into another well. This will be essential in manipulations involving augmentation by bacteria to enhance bioremediation.

Figure 4.3. FAME chromatogram showing chromatographic column retention times and peak heights of a microorganism isolated from subsurface rock cores at Idaho National Engineering and Environmental Laboratory INEEL-10 test site. The 1.593 (far left) peak is the solvent peak. Remaining are carbon fatty acid peaks. All of these constitute a unique profile that can then be compared to those in the FAME database. This organism has a high similarity index to *Bacillus atrophaeus*. Image Courtesy of Tamas Torok, Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory.



## Aerobic Respiration

Aerobic respiration is very efficient because  $O_2$  has a very positive redox potential, leading to a large difference in net reduction potentials between the primary electron donor and terminal electron acceptor. This means a greater release of energy and the synthesis of more ATP.

Aerobic chemolithoautotrophs can use carbon dioxide as their sole carbon source but also generate energy from inorganic compounds (electron donors) with oxygen as an electron acceptor. In aerobic respiration, compounds such as reduced iron ( $Fe^{2+}$ ), ammonium sulfide ( $(NH_4)_2S$ ), or molecular hydrogen ( $H_2$ ), can act as electron donors. These reactions hold promise for bioremediation as they can determine the fate and transport of radionuclides and other metals. For example, when dissolved  $Fe^{2+}$  is oxidized to  $Fe^{3+}$ , hydrous iron-oxide mineral precipitates are formed. These precipitates provide surfaces for reactions with other metals and radionuclides, allowing complexation to occur with contaminants, and thereby changing contaminant mobility. This will make the contaminant less likely to enter groundwater.

## Anaerobic Respiration

The reactions collectively known as anaerobic respiration are defined by their electron acceptor. The major modes of anaerobic respiration are denitrification, sulfate reduction, and ferric iron reduction.<sup>4</sup> The processes of methanogenesis and fermentation may also be important in anaerobic environments. Some of the microorganisms that use these compounds as electron acceptors can also use metals and radionuclides (such as chromium and uranium) as terminal electron acceptors. However, because none of these electron acceptors have as large a reduction potential as the  $O_2/H_2O$  couple (Table 4.1), less energy is released when they are used.

When inorganic compounds such as nitrate ( $NO_3^-$ ), sulfate ( $SO_4^{2-}$ ), and carbon dioxide ( $CO_2$ ) are reduced for use as nutrient sources, they are said to be assimilated, and the reduction process is called assimilative metabolism. When they are used only for energy metabolism as electron

acceptors, this process is called dissimilative metabolism. In assimilative metabolism only enough of the compound is reduced to satisfy the nutritional needs, and the reduced atoms are converted to cell material. In dissimilative metabolism, a relatively large amount of the electron acceptor is reduced, and the reduced product is excreted into the environment. The focus of this section is on dissimilatory processes.

**Nitrate reduction (Denitrification).** Basically, denitrification is the dissimilative reduction of nitrate ( $NO_3^-$ ) to nitrogen gas ( $N_2$ ), which the microbes couple to oxidation of a substrate to gain food for growth. This is a two-step process. The first step is the reduction of  $NO_3^-$  to nitrite ( $NO_2^-$ ). This is catalyzed by the enzyme nitrate reductase. The next step is the reduction of  $NO_2^-$  to  $N_2$ . This is catalyzed by nitrite reductase and follows one of two paths: either through nitric oxide (NO) or nitrous oxide ( $N_2O$ ).

If oxygen is removed from a system and nitrate is present, denitrification will occur to the exclusion of most other metabolisms. Denitrification provides microbes with a relatively high amount of energy, and microbial growth rates are consequently high compared to other anaerobic metabolisms.

Under some conditions, the first step in the redox reaction (reduction of nitrate to nitrite) is faster than the second, and this disparity may cause the buildup of nitrite, which is inhibitory to many bacteria. Thus, denitrifiers may be important to biological treatment of metals and radionuclides by inhibiting the activity of dissimilatory iron reduction or sulfate reduction, causing an increase in pH or depleting substrate. Denitrifiers can be integral to an in situ biological treatment approach if nitrate is one of the contaminants.

Most denitrifiers are facultative aerobes, that is, they can switch to denitrification when  $O_2$  is no longer available as an electron acceptor. The bacteria *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa* are three such denitrifiers.

4. Many of the microorganisms involved in anaerobic respiration are extremophiles — they can exist at extremely hot temperatures, in salty bodies of water, and in environments with extreme variations in pH. For more information, see the feature “Extremophiles: Microscopic Exotica” on page 34.

**Iron reduction.** The reduction potential of  $\text{Fe}^{3+}/\text{Fe}^{2+}$  is very electropositive (Table 4.1). Several microorganisms are able to couple oxidation of hydrogen or organic compounds to the reduction of  $\text{Fe}^{3+}$  and gain energy for growth. These bacteria include species from several genera, including *Geobacter*, *Desulfuromonas*, *Pelobacter*, *Shewanella*, *Ferrimonas*, *Geovibrio*, *Geothrix*, and *Bacillus*. These organisms have a broad spectrum of other metabolic capabilities as well. For instance, *Shewanella* species can use oxygen, nitrate, uranium, manganese, and iron as electron acceptors.

The use of  $\text{Fe}^{3+}$  and other metals by certain microbial groups as terminal electron acceptors for anaerobic respiration is of particular relevance to bioremediation of heavy metals and radionuclides. Dissimilatory iron reducers and other microorganisms can reduce mineral-associated iron to produce reactive sites within the minerals or to directly reduce contaminants, such as uranium and chromium. A number of species are able to reduce structural iron, even in amorphous minerals such as ferrihydrite, and crystalline iron oxy-hydroxides, including the minerals hematite, goethite, and magnetite.

**Sulfate reduction.** Sulfate ( $\text{SO}_4^{2-}$ ) is the most common sulfur compound used as an electron acceptor in dissimilative sulfate reduction. Sulfate reduction produces much less energy, however, than  $\text{O}_2$  or  $\text{NO}_3^-$  (Table 4.1), and growth yields are lower. The first product of sulfate reduction is sulfite ( $\text{SO}_3^{2-}$ ). The end product is hydrogen sulfide ( $\text{H}_2\text{S}$ ). Usually, organic carbon compounds are the primary electron donors in sulfate reduction. But in some cases hydrogen gas ( $\text{H}_2$ ) can be an inorganic electron donor. Sulfate-reducing microbes that grow using  $\text{H}_2$  as an electron donor are chemolithotrophs. However, most sulfate-reducing organisms are chemoorganotrophs, using various organic compounds as electron donors, including the fermentation (see below) products lactate, acetate, and ethanol.

The metabolic activity of sulfate reducers is not limited to the reduction of sulfate; other metals can be reduced by these organisms. Furthermore, sulfate reduction and the direct reduction of iron

can occur simultaneously, depending on how available the iron is to microbial reduction. *Desulfovibrio desulfuricans* is a well-known sulfate-reducing bacterium that can also use iron, uranium, or chromium as an electron acceptor.

**Methanogenesis.** Methanogenesis is the microbial production of methane ( $\text{CH}_4$ ) through the reduction of  $\text{CO}_2$  (Table 4.1). Carbon-dioxide reduction is coupled to oxidation of hydrogen, with hydrogen gas ( $\text{H}_2$ ) being one of the most common electron donors. Organic compounds such as acetate, formate, and trimethylamine can also be electron donors. Methanogens are archaea. These microorganisms are present in most anaerobic environments, including waterlogged sediments, marshes, rice paddies, and the gastrointestinal tracts of some animals. The microorganisms in cows are prolific methane producers. Although these reactions probably do not directly impact metals or radionuclides, they may have an indirect and possibly adverse effect by competing for substrates with dissimilatory iron reducers or sulfate reducers (which can catalyze reactions that affect inorganic contaminants). However, under many conditions relevant to in situ treatment of metals and radionuclides, the dissimilatory iron-reducing and sulfate-reducing microorganisms can successfully out-compete methanogens for the substrates.

**Fermentation.** Fermentation is an anaerobic process in which energy generation occurs by redox reaction and in which an organic substrate serves as both electron donor and electron acceptor. The organic compound, such as a sugar or amino acid, is broken down into smaller organic molecules, which accept the electrons that were released during the breakdown of the energy source. Although metals and radionuclides are not directly affected by fermentation, it can be an important step in the production of substrates used by dissimilatory iron-reducing and sulfate-reducing bacteria, which are the primary catalysts of reactions that affect inorganic contaminants. In addition, there is evidence in sediments that fermentation products can serve as metal complexing agents, increasing metal contaminant mobility.

## EXTREMOPHILES: MICROSCOPIC EXOTICA

Life can be found almost everywhere on this planet. Much of this life exists in the form of microbes. And the most exotic microorganisms, extremophiles, can live in niches where no other organisms are found. Thermophiles characteristically grow at temperatures greater than 45°C (113°F). Hyperthermophiles can live in environments with temperatures of 80°C or higher. Some extremophiles can tolerate pH levels less than two or greater than ten. And halophiles exist in saturated saline. A number of these microbes belong to the newly defined domain of life, the Archaea. Scientists first believed extremophiles were predominantly archaea, but now they are starting to see bacteria in these extreme environments as well. For example, samples taken from Yellowstone Park's Obsidian Pool showed the ratio of thermophilic bacteria to archaea as 50 to 1 (Pace, 1997). This hot-temperature environment is high in hydrogen sulfide, iron, hydrogen, and carbon dioxide.

Extremophiles can be a boon to bioremediation. Many extreme environments are anaerobic, so these microbes do not need oxygen. They can survive environments that are similar to toxic waste sites and would poison or kill other organisms. Proteins from some of these extremophiles are presently being isolated and characterized in the hopes of learning how they function in such extreme environments. Hopefully, this information will be helpful in re-engineering other microorganisms so that they can tolerate extreme conditions.

Below are profiles of several interesting extremophiles.

***Methanococcus jannaschii*** was the first archaeon whose genome was sequenced (Bult et al., 1996). It was first isolated at the base of a Pacific thermal vent off the coast of Baja California in 1983. *M. jannaschii* possesses a small (about 1.66 Mbp) genome. It is a methanogen (methane producer) and a thermophile. This microbe normally lives at about 8,000 feet below sea level, where the pressure equals about 230 atmospheres (3,380 pounds per square inch). It is strictly anaerobic and autotrophic.

***Bacillus infernus*** (the "bacillus from hell") is a newly identified species of bacteria (Boone et al., 1995). This is the first-known strictly anaerobic member of the bacterial genus *Bacillus*, which prior to this had always been described as aerobic. This thermophile was obtained from a depth of about 9,000 feet below the land surface. Microbes at this depth have been in isolation from the surface for millions of years and have evolved very exotic metabolisms and slow rates of reproduction.

***Deinococcus radiodurans*** species can withstand exposure to radiation levels up to 1.5 million rads (500 rads is lethal to humans). At that point its chromosomes shatter into hundreds of fragments. It is believed that *D. radiodurans* has a more active DNA-repair mechanism than other microorganisms because the conditions under which it is able to survive are so damaging to other species. It isn't exactly clear how *D. radiodurans* obtained its remarkable radiation resistance. It was first observed in the 1950s in cans of meat that had been exposed to supposedly sterilizing doses of radiation. The microbe has certain possibilities for bioremediation. Conceivably, a strain of *D. radiodurans* modified with genes from other organisms having bioremediation ability could be used to treat highly radioactive waste.

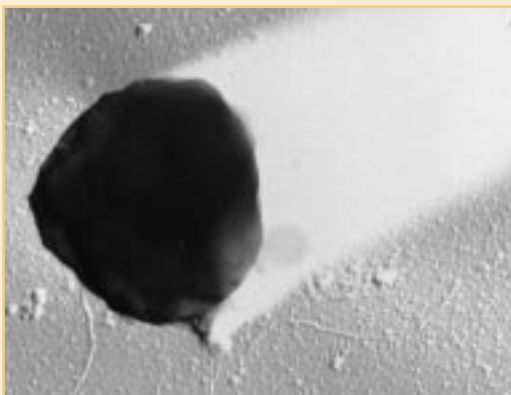


Figure 4.4. *Methanococcus* species. K. O. Stetter, Universität Regensburg, Faculty of Natural Sciences.



Figure 4.5. New species *Bacillus infernus*, “the bacillus from hell,” magnified 50,000 times. Transmission electron micrograph taken by Henry C. Aldrich, University of Florida.

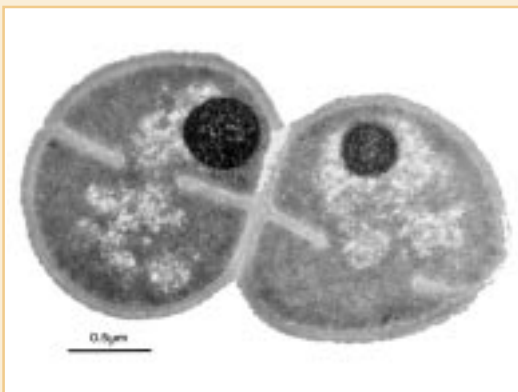


Figure 4.6. *Deinococcus radiodurans*, magnified 60,000 times. Taken by John Battista and Peggy O’Cain of Louisiana State University.

## MICROBIAL CONSORTIA

Microbial biotransformation and biodegradation can occur only if microorganisms are present that can metabolize the contaminant. In particular, there must be microbial enzymes that can act as catalysts for the oxidation–reduction reactions that will degrade or transform the compound. However, knowing and capitalizing on the relationship of the organisms to the substrate is only one aspect of bioremediation. Another aspect is understanding the interrelationships of the microorganisms in the microbial community, or consortium. A consortium is a relatively stable but loose-knit association of microorganisms in an environment. Microbe-to-microbe interactions are complex, and may run the gamut from symbiosis to predator–prey relationships.

One type of microbial consortium is a biofilm (Figure 4.7). Biofilms are created by groups of microorganisms adhering to a sediment particle or other surface and releasing exopolysaccharides. In fact, this phenomenon may create small “stagnant” areas in the sediment pore spaces where all of the oxygen is depleted, even though the fast groundwater flow path areas and therefore the bulk environment are saturated with oxygen or are aerobic. These biofilms also allow organisms to come into juxtaposition so that a variety of complex relationships, as discussed below, can develop.

In symbiosis, two species form an association whereby the individuals of one or both species are benefited. Two of the most common symbiotic relationships are commensalism and mutualism. Commensalism is a symbiotic relationship in which a one-sided association is formed between two species. The individuals of one provide sustenance to those of the other. Neither group, however, is harmed. In mutualism, both species benefit from each other’s products.

Syntrophy (“mutual feeding”) is a well-known form of mutualism in which members of two species are nutritionally dependent on one another. In a syntrophic relationship, the organisms together can degrade a substance that neither can degrade separately. For example, in the coupled reaction of ethanol fermentation with methanogenesis, a syntrophic relationship is formed between an ethanol fermenter and a methanogen. The ethanol fermenter produces hydrogen ( $H_2$ ) and acetate, but the energy yield from that reaction is low. The methanogen then consumes the  $H_2$  from the fermentation half reaction to produce methane with a relatively high energy yield. The coupled reaction produces a higher energy yield for the fermentation half reaction. Therefore, the ethanol fermenter

utilizes more of the ethanol in the coupled reaction than it would without the syntrophic relationship with the methanogen. And the methanogen gets the  $H_2$  it needs to produce the methane.

In predator–prey relationships, the first microbe consumes a substrate, and then the second microbe consumes the first microbe. The interactions of bacteria and protozoa (unicellular eukaryotic microorganisms) are an example of such a relationship. Protozoa that consume bacteria and excrete material that is readily utilizable by the same or other bacteria in the biofilm can have a dramatic effect on the rate and type of biodegradation/bioremediation. High rates of protozoa predation at sites being bioremediated by injection of bacteria could decrease the effectiveness of the treatment. However, other sites may benefit from high rates of predation by increasing turnover rate and thereby the biodegradation rate of the contaminant. High rates of predation may also lower the overall numbers of bacteria, even though the activity has increased. This gives the false impression that bacterial densities have decreased and therefore the bioremediation of that subsurface environment has declined. Other relationships between two species can also have both positive and negative effects on bioremediation.

In most situations, the microbes capable of metabolizing the contaminant are already present in the targeted area. However, if the contamination is recent or if the contaminants are complex, anthropogenic compounds (xenobiotics), or compound mixtures, there is a greater chance that capable microorganisms will not be present. And even if the right microbes are present, there may not be enough for a successful cleanup. This can be due to environmental conditions unsuitable for microbial proliferation and activity within the desired time frame to comply with government regulations.

If that is the case, commercially available microbial inoculants can be added through bioaugmentation (discussed in Section II). Inoculants usually consist of a sample of this microbial community that is extracted and cultivated in the laboratory. Conditions are then manipulated ex situ to encourage the growth of the suitable microbes, and then the conditions are duplicated in the field. In this way, organisms that are dormant or are in insufficient quantities but are specifically suited for the bioremediation of a particular contaminant can be selected. One of the major challenges to bioaugmentation is survival of the introduced microorganisms in the contaminated environment. Native or indigenous microbes may out-compete the introduced organisms for limited nutrients.

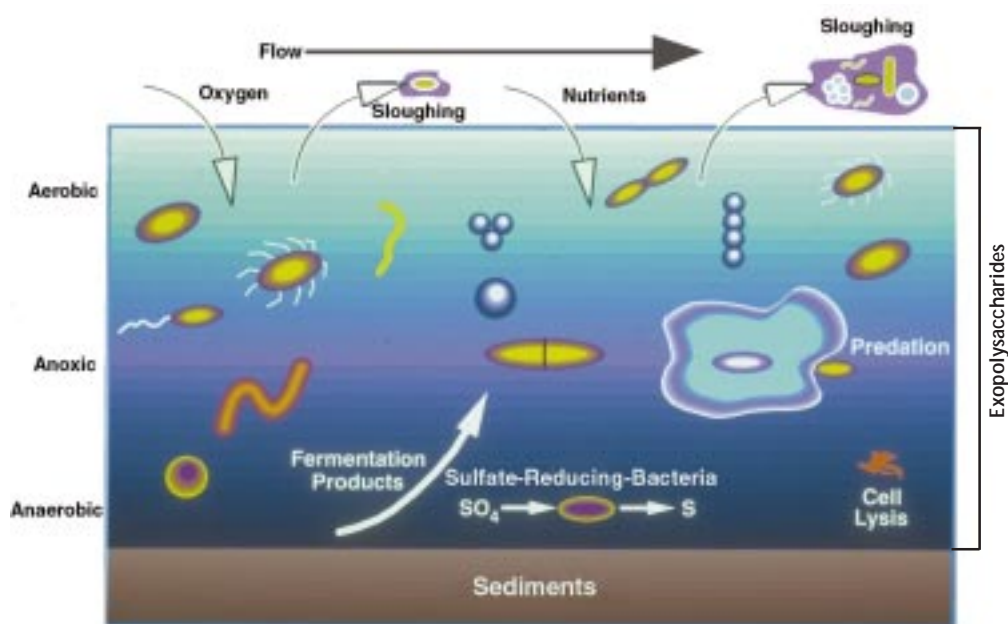


Figure 4.7. Mature biofilm.